Superior Suppressive Capacity of Skin Tregs Compared with Lung Tregs in a Model of Epicutaneous Priming

Subhashree Mahapatra[1,4], Melanie Albrecht[1,4], Abdul M. Baru[2], Tim Sparwasser[2], Christina Herrick[3] and Anna M. Dittrich[1]

We have previously shown that T helper type 2 (Th2)-polarized airway inflammation can facilitate priming to new antigens in the lungs, which we called "collateral priming". To investigate whether allergic skin inflammation can also facilitate priming toward new antigens, we developed an allergic skin inflammation model based on an allergic lung inflammation model. Mice were sensitized intraperitoneally toward the primary antigen, ovalbumin. Challenge was subsequently performed intranasally or epicutaneously with ovalbumin and a secondary antigen, keyhole limpet hemocyanin (KLH). Re-challenge consisted of local application of either antigen alone. Analysis of KLH-specific antibody responses, KLH-specific cytokines, and local inflammation demonstrated tolerance induction toward the secondary antigen in the skin, whereas in the lung priming had occurred. Flow-cytometric analysis revealed increased numbers of regulatory T cells (Tregs), increased cytotoxic T lymphocyte antigen-4 (CTLA-4) expression, and an enhanced suppressive capacity of Tregs from skin-draining lymph nodes when compared with Tregs from the lung-draining lymph nodes. Furthermore, depletion of Tregs resulted in restoration of collateral priming in the skin. These results demonstrate crucial local differences between the Treg function in the skin and lung to repetitive antigen exposure, which can decisively influence the immune response toward new antigens.

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INTRODUCTION

Polysensitization is a clinical phenomenon where a person is sensitized to more than one allergen family and has an increased susceptibility to develop new allergies, which can occur in the various organs involved with allergic disease manifestations (Fasce *et al.*, 2004; Ciprandi *et al.*, 2008). Polysensitization has been modeled in mice as a process where ongoing airway inflammation facilitates priming to secondary unrelated antigens, which has been termed "collateral priming" (Eisenbarth *et al.*, 2004; Hayashi *et al.*, 2005; Blumchen *et al.*, 2006; Cadot *et al.*, 2010; van Rijt *et al.*, 2011, 2012). Collateral priming depends on adaptive immune responses and IL-4 (Dittrich *et al.*, 2008), modulating T cell, dendritic cell (Dittrich *et al.*, 2008), and epithelial cell function (Albrecht *et al.*, 2012), and also occurs in T helper type 1 (Th1) and Th17 polarized airway inflammation (Albrecht *et al.*, 2011). However, studies addressing immunological mechanisms involved in polysensitization in other organ systems, such as the skin or the gut, are lacking.

In this current study, we compared collateral priming in a mouse model of allergic skin inflammation with a model of allergic lung inflammation. We could demonstrate that, in contrast to an ongoing pulmonary inflammation, an ongoing skin inflammation does not facilitate priming to a secondary, unrelated antigen. We identified differences in Tregs from the skin versus lung-draining lymph nodes (dLNs) and demonstrated restoration of collateral priming by depletion of Tregs prior to EC antigen contact. This pre-clinical data underline the therapeutic potential of repetitive allergen "desensitization" via the skin to induce tolerance.

RESULTS

Collateral priming for antigen-specific antibody responses observed only in the lung collateral group

To determine sub-immunogenic doses of two antigens (ovalbumin (OVA) and keyhole limpet hemocyanin (KLH)),

[1]*Department for Pediatric Pulmonology, Allergology and Neonatology, Medical School Hannover, Hannover, Germany;* [2]*Institute of Infection Immunology, TWINCORE, Centre for Experimental and Clinical Infection Research, Hannover, Germany and* [3]*Department of Dermatology, Yale University School of Medicine, New Haven, Connecticut, USA*

Correspondence: Anna-Maria Dittrich, Department for Pediatric Pulmonology, Allergology and Neonatology, Medical School Hannover, Carl-Neuberg-Str. 1, Hannover 30625, Germany. E-mail: Dittrich.Anna-Maria@MH-Hannover.de

[4]*These authors contributed equally to this work.*

Work was performed at Hannover Medical University, Hannover, Germany.

Abbreviations: AD, atopic dermatitis; BAL, bronchoalveolar lavage; CTLA-4, cytotoxic T lymphocyte antigen-4; DEREG, depletion of regulatory T cell; dLN, draining lymph node; EC, epicutaneously; EPIT, epicutaneous immunotherapy with allergen; IN, intranasally; IP, intraperitoneally; KLH, keyhole limpet hemocyanin; OVA, ovalbumin; Th, T helper cell; T_{resp}, responder T cell; Treg, regulatory T cell

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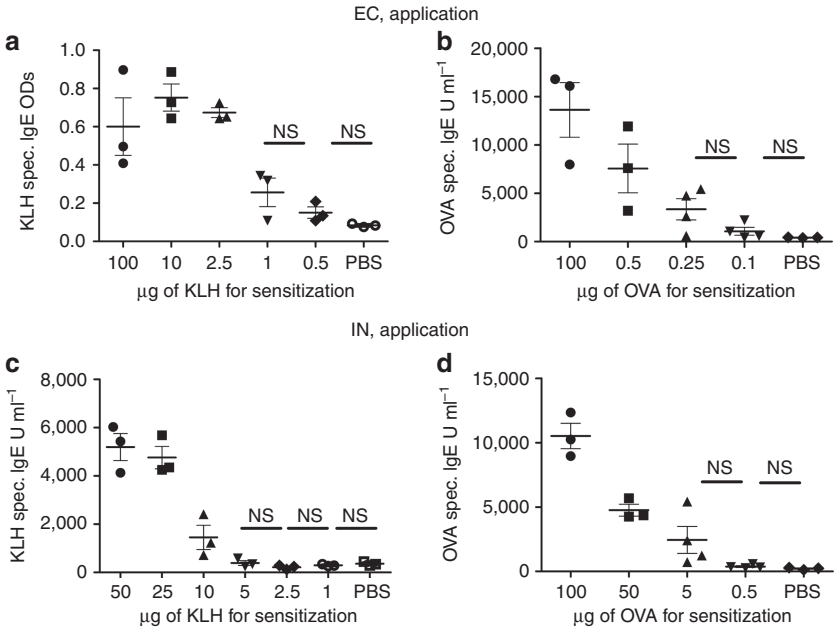


Figure 1. Titration of sub-immunogenic doses. Animals were sensitized as outlined in the materials and methods section and challenged epicutaneously (EC) (**a** and **b**) or intranasally (IN) (**c**, **d**) with the indicated doses. Ovalbumin (OVA)- (**b** and **d**) and keyhole limpet hemocyanin (KLH)- (**a** and **c**) specific IgG1 and IgE were determined in the sera of the mice by ELISA. $n = 3$ animals per group. Representative study from $n = 5$ studies for KLH and $n = 2$ studies for OVA. NS, not significant.

we performed titration experiments as shown in Figure 1. For KLH, a dose of 1 µg via the epicutaneous (EC) route proved insufficient to induce immunoglobulin secretion (Figure 1a) and for intranasal (IN) application a dose of 5 µg or less was sub-immunogenic (Figure 1c), whereas for OVA the sub-immunogenic dose was from 0.25 µg EC (Figure 1b) and from 5 µg IN (Figure 1d). Similarly, we used these experiments to gauge doses for a clear-cut positive response. We proceeded to develop a skin collateral priming model to compare collateral priming in the skin with an established lung collateral priming model.

We chose an OVA IP sensitization model that allows for the same route of primary sensitization in both organ systems. It has previously been utilized to study collateral priming in the lungs (Eisenbarth *et al.*, 2004) and was adapted from established skin models (Spergel *et al.*, 1998; Herrick *et al.*, 2003) by us. KLH was chosen as the second antigen. Accordingly, we designated the following study groups: the O/O group (OVA upon primary and secondary local challenge), the O+K/K group (OVA and KLH upon primary local challenge and KLH alone upon re-challenge), and the K/K group (KLH upon challenge and re-challenge) (Figure 2a).

OVA-specific IgG1 and IgE were elevated in the serum of all the groups, confirming sensitization toward OVA as a primary antigen (Figure 2b and c). We observed increased KLH-specific IgG1 and IgE in the lung collateral priming group (O+K/K), confirming collateral priming toward KLH in the lung group (Figure 2d and e). However, in the skin collateral priming group (O+K/K), we could not detect induction of either KLH-specific IgG1 or IgE, demonstrating a lack of B-cell priming toward KLH in the skin (Figure 2d and e).

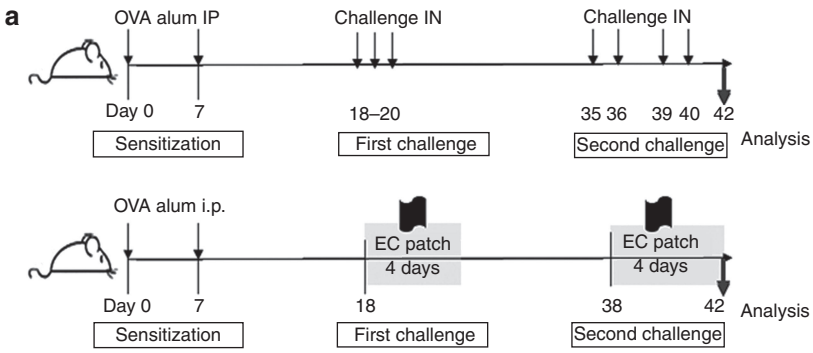
To exclude antigen-dependent effects, we performed consecutive cross-over experiments where KLH was utilized as the primary antigen for IP sensitization and OVA was used as the secondary antigen (Figure 2f and g). Similarly to what we had previously observed, IN OVA administration at sub-immunogenic doses led to increased OVA-specific IgG1 and IgE in the collateral priming group (lung O+K/O), whereas EC OVA administration at sub-immunogenic doses did not induce OVA-specific IgG1 or IgE (skin O+K/O).

Collateral priming for antigen-specific T-cell cytokine secretion observed only in the lung collateral priming group

Subsequently, we compared T-cell polarization toward KLH by re-stimulating cells from dLNs with KLH.

Analysis of IL-4 secretion upon re-stimulation with OVA confirmed T-cell priming and polarization toward the primary antigen OVA in the O/O and O+K/K groups (Figure 3a). However, the detection of IL-13 (Figure 3b) and IFN-γ (Figure 3c) secretion in all the groups may reflect induced secretion of IL-13 in skin dLNs post epicutaneous contact with an antigen (Herrick *et al.*, 2003).

Upon re-stimulation with KLH, we observed significantly elevated levels of IL-4 and IL-13 in the lung collateral priming group in comparison with all other groups. This confirmed the occurrence of collateral priming (Figure 3d and e, lung). In contrast, we did not observe increased levels of the Th2 cytokines in the skin collateral priming group (O+K/K) when compared with the other skin groups (O/O, K/K, Figure 3d and e, skin). Secretion of the Th1 cytokine IFN-γ was not



Group	Sensitization	First challenge	Second challenge
Positive	20 μg OVA	i.n. : 25 μg OVA e.c. : 100 μg OVA	i.n. : 25 μg OVA e.c. : 100 μg OVA
Collateral priming		i.n. : 25 μg OVA+5 μg KLH e.c. : 100 μg OVA+1 μg KLH	i.n. : 25 μg KLH e.c. : 100 μg KLH
Negative		i.n. : 5 μg KLH e.c. : 1 μg KLH	i.n. : 25 μg KLH e.c. : 100 μg KLH

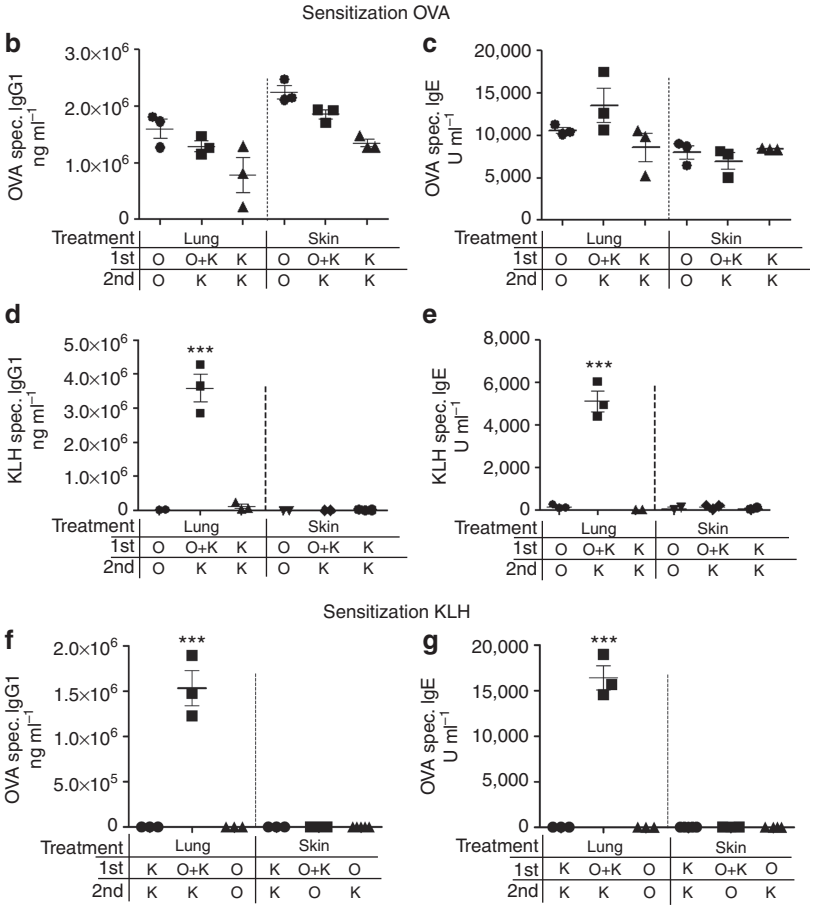


Figure 2. Antigen-specific antibody response directed toward the secondary antigen observed only in the lung collateral group. (**a**) Schematic overview of collateral priming protocol. (**b** and **c**) Ovalbumin (OVA)-specific IgG1 and IgE. (**d** and **e**) keyhole limpet hemocyanin (KLH)-specific IgG1 and IgE levels detected in the sera of the mice by ELISA. (**f** and **g**) OVA-specific IgG1 and IgE levels detected in the sera of mice that had undergone a cross-over collateral priming treatment with KLH used as primary and OVA as secondary antigen (doses for cross-over experiment are indicated in the Materials and Methods section); detection by ELISA; $n = 3$ animals per group. ***$P < 0.001$. Representative study from $n = 2$ to 5 studies. EC, epicutaneous; IN, intranasal; IP, intraperitoneal.

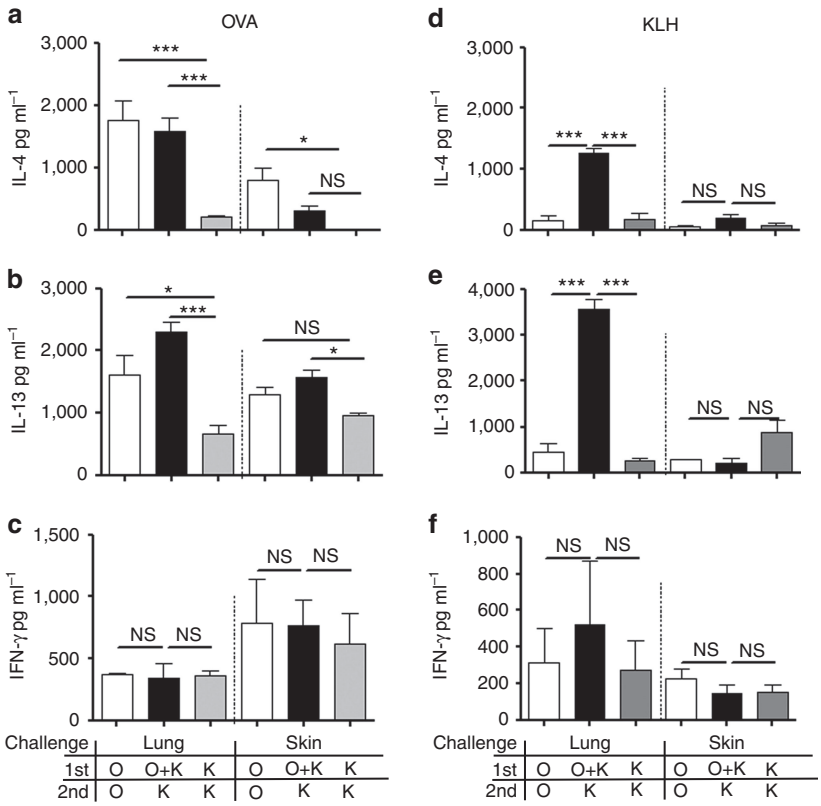


Figure 3. Antigen-specific T-cell cytokine secretion directed toward the secondary antigen observed only in the lung collateral priming group. Detection of antigen-specific IL-4, IL-13, and IFN-γ secretion in the supernatants of draining lymph node (dLN) cells by ELISA after *ex vivo* re-stimulation with ovalbumin (OVA) (**a**–**c**) or keyhole limpet hemocyanin (KLH) (**d**–**f**) for 72 hours. Depicted are cultures of LN cells from individual mice with $n = 5$ animals per group. ***$P < 0.001$ and *$P < 0.05$. Representative study out of $n = 4$. NS, not significant.

significantly different between the groups (Figure 3f). In aggregate, these results demonstrated a lack of KLH-specific priming of T cells in the skin via collateral priming.

Local inflammation was not observed in the skin collateral group

In the bronchoalveolar lavage (BAL) of the lung collateral priming group (O+K/K), cell counts were significantly increased when compared with the negative control group (K/K) but were similar to the positive control group (O/O; Figure 4a). The BAL cellular influx consisted mainly of eosinophils, which were undetectable in the negative control group (Figure 4b). In addition, the levels of monocytes, lymphocytes, and neutrophils were increased in the two groups (Supplementary Figure S1a–c online).

However, in the skin-positive control group (O/O) epidermal thickening, lymphocytic influx in the dermis, and mast cell staining were much more pronounced than in either the collateral priming group (O+K/K) or the negative control group (K/K; Figure 4d and e). With this, there was a statistically significant difference in the mast cell counts (Figure 4c). Overall, these data confirmed a lack of local inflammation in the skin collateral priming group as compared with the lung.

Increased proportion and numbers of Tregs observed in skin dLNs

Several studies have highlighted a central role of Tregs in skin inflammation (Ghoreishi *et al.*, 2009; Rosenblum *et al.*, 2011; Gomez de *et al.*, 2012; Lehtimaki *et al.*, 2012). However, as their role in the lack of collateral priming in the skin had not been defined, we decided to address this. For this purpose, we stained for CD4+Foxp3+ cells (Tregs) in dLNs. Flow cytometric analysis of the dLNs in the skin revealed that not only repetitive exposure to the primary antigen OVA (i.e., O/O and O+K/K groups) but also to the secondary antigen KLH (i.e., K/K/group) resulted in significantly increased Treg numbers and percentages (Figure 5a and b, denoted by $). However, in the lung, repetitive antigen contact elicited only a significantly increased Treg number in the O+K/K group (Figure 5b, denoted by $). Strikingly, we always observed significantly higher Treg percentages and numbers in the animals exposed EC as compared with the animals of the respective group exposed IN (Figure 5a and b, denoted by #). Regarding Treg percentages, this also held true for the comparison between naive (−/−) animals in the skin and the lung groups (Figure 5a, denoted by #).

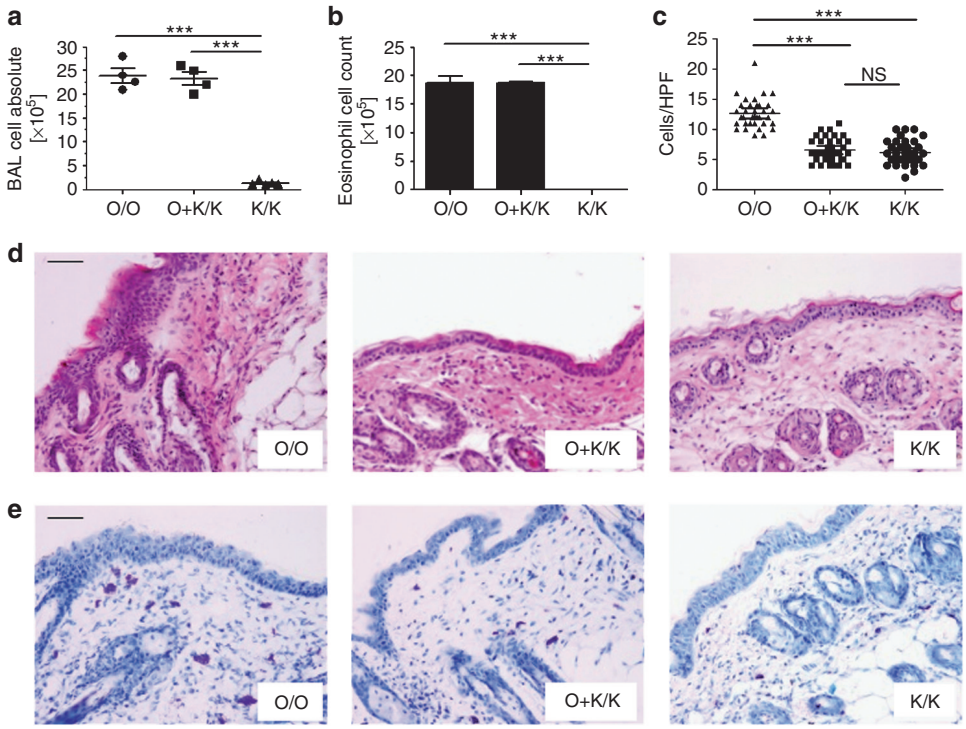


Figure 4. Local inflammation observed only in the lung collateral priming group. (**a** and **b**) Absolute cell count, eosinophil count in bronchoalveolar lavage (BAL) fluid. (**c**) Thirty-five high-power fields from 7 slides/group were counted in a blinded manner for statistical assessment of mast cell counts in skin sections. (**d** and **e**) Hematoxylin and eosin (H&E) and toluidine blue staining of the antigen-challenged skin. Original magnification is ×200. Scale bar = 50 μm. $n = 4$ animals per group. ***$P < 0.001$. Experiment is representative of $n = 3$.

Increased CTLA-4 expression in skin dLNs

Cytotoxic T lymphocyte antigen-4 (CTLA-4) has been implicated in conferring Treg-mediated suppression (Sansom and Walker, 2006). We measured CTLA-4 expression with flow cytometry by analysis of mean fluorescence intensity of CTLA-4 from dLNs. This revealed that CTLA-4 expression was significantly higher on skin dLN Tregs from both the collateral priming group (O+K/K) and the animals that had undergone two OVA challenges (O/O), when compared with the expressions in their respective lung groups (O+K/K O/O, Figure 5c, denoted by #). Similarly to what we had observed with FoxP3+ cell numbers in the lung groups, antigen treatment did not increase CTLA-4 expression when compared with naive controls (−/−; Figure 5c, lung). In contrast, the skin groups treated with the primary antigen OVA showed significantly increased CTLA-4 expression for both the positive control (O/O) and the collateral priming group (O+K/K), when compared with the naive controls (Figure 5c, skin, denoted by $).

Tregs from skin dLNs display enhanced suppressive activity

We performed suppression assays to evaluate the suppressive capacity of Tregs from the two organ systems' dLNs. Sorted Tregs from depletion of regulatory T cells (DEREG) mice (Lahl *et al.*, 2007) submitted to the lung or skin collateral priming treatment protocols (Figure 2a) were co-cultured with labeled CD4+ responder T cells (T_{resp}) induced to proliferate by plate-bound CD3/CD28. Analysis of VP-450 dilution within the T_{resp} population via flow cytometry after 72 hours showed that ~59% of T_{resp} had been induced to proliferate at this time point (Figure 6a, left graph). Analyses of T_{resp} proliferation in co-culture with Tregs revealed that both skin and lung dLN–derived Tregs inhibited T_{resp} proliferation (Figure 6a, middle and right graph). Of note, at the 8:1 ratio (T_{resp}:Tregs), this inhibition was significantly greater for Tregs from the skin dLNs (~17% proliferation) versus Tregs from the lung dLNs (~37% proliferation; Figure 6a, middle and right graph). Taken together, these results demonstrate that skin dLN Tregs are not only more numerous after antigen contact but also confer more efficient suppression of proliferation compared with dLN Tregs isolated from the lungs.

Depletion of Tregs restores EC priming toward a secondary antigen

We depleted Tregs from DEREG mice before the first challenge in our EC model (Figure 2a). Flow-cytometric analysis of blood samples 48 hours after the first EC antigen contact demonstrated sufficient depletion of Tregs (Figure 6d, left panel), whereas analyses of blood samples 48 hours after the second EC antigen contact demonstrated repopulation with Tregs (Figure 6d, right panel). Comparison of immunoglobulin secretion by diphtheria toxin-treated mice versus phosphate-buffered saline (PBS)-treated controls after the complete EC collateral priming protocol (Figure 2a) revealed

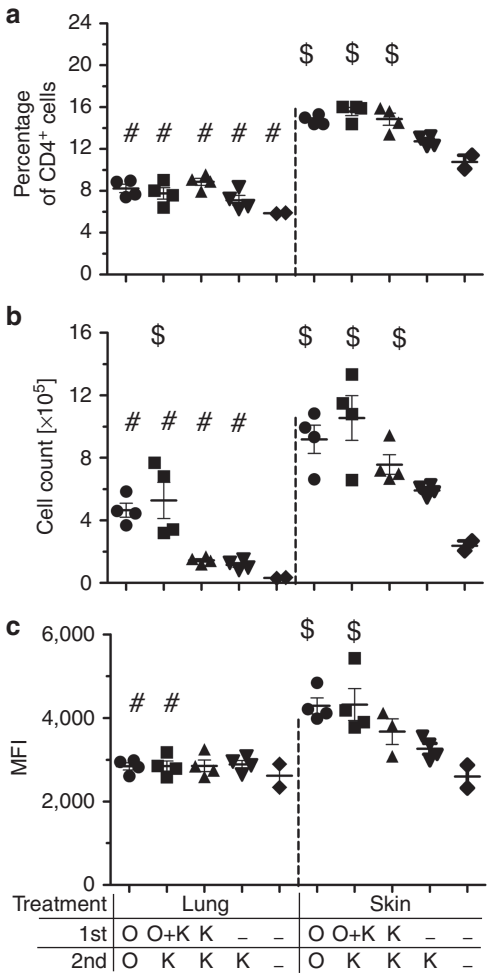


Figure 5. Increased percentage of regulatory T cells (Tregs) and increased expression of cytotoxic T lymphocyte antigen-4 (CTLA-4) on Tregs in skin-draining lymph nodes (dLNs). (**a** and **b**) Percentage of Foxp3$^+$ cells among CD4$^+$ cells as identified by FACS staining and total numbers of CD4$^+$Foxp3$^+$ cells in dLNs determined by multiplication of dLN cell numbers with percentages of Foxp3$^+$ positive cells among total cells. (**c**) Comparison of mean fluorescence intensity (MFI) for CTLA-4 staining on CD4$^+$ Foxp3$^+$ cells in dLNs. $n=2$–3 animals per group. $ denotes significantly different (at least $P<0.05$) compared with naive controls (−/−) of the same organ. # denotes significantly different (at least $P<0.05$) compared with respective treatment group in the EC protocol. Representative experiment of $n=3$.

a significant secretion of KLH-specific IgG1 and IgE only in the Treg depleted group (Figure 6e and f). These results suggest that the presence of Tregs contributed to the lack of collateral priming that we had observed in the EC model.

DISCUSSION

Our data suggest that an ongoing skin inflammation does not facilitate priming to a secondary, unrelated antigen. Thus, our results starkly contrast the previous findings observed in models for allergic lung inflammation (Eisenbarth *et al.*, 2004; Hayashi *et al.*, 2005; van Rijt *et al.*, 2011) but are in line with studies from Rosenblum *et al.* (2011). These had shown that, upon expression of a self-antigen in the skin, regulatory T cells could become activated, proliferate, and differentiate into potent suppressors.

We cannot completely exclude that differences in the doses of the antigens underlie the differences we observed, given the knowledge on antigen- and dosage-dependency of tolerance induction in the skin (Cavani, 2008). However, we believe that our technique of careful titration allowed us to select equivalent antigen doses for both antigens and supported our approach to use different doses of the antigens. Our cross-over experiments supported our view that the lack of collateral priming in the skin is antigen independent, with Treg numbers and their capacity being affected differently by repetitive antigen exposure in the skin versus lung.

It has been acknowledged for some time that Tregs have a decisive role in the suppression of skin inflammation (Ramsdell and Ziegler, 2014). Furthermore, in models of atopic dermatitis (AD) and contact hypersensitivity, Lehtimaki *et al.* (2012) showed that repetitive EC contact increases the number of Tregs in the skin, and depletion of these cells aggravates the AD and the contact hypersensitivity phenotype (Fyhrquist *et al.*, 2012). These results are in accordance with our own findings. Furthermore, depletion of Tregs during the sensitization phase can aggravate allergic airway inflammation (Baru *et al.*, 2010), whereas depletion during the challenge phase has no such effect (Baru *et al.*, 2012). Similarly, we could retrace these findings in our own depletion experiments: immunoglobulin responses toward the primary antigen (OVA) were not influenced by depletion of Tregs during the challenge phase (Supplementary Figure S3 online), but depletion of Tregs during EC sensitization with the secondary antigen allowed collateral priming to take place in the skin. In addition, our own data provide a comparison of the *ex vivo* suppressive capacities of the skin versus lung Tregs from the collateral priming groups (O +K/K, Figure 6a–c). Our data suggest that the functional differences we observed might be attributable to differences in CTLA-4 expression (Figure 5c, denoted by #) and also quantitative differences of Tregs (Figure 5a and b, denoted by #).

Different mechanisms might lead to the qualitative and quantitative differences between Tregs in the skin and lung. In steady-state human peripheral blood, the majority of Tregs express CLA, a skin homing receptor (Hirahara *et al.*, 2006). This suggests that skin homing of Tregs constitutes a preferred route of Treg trafficking under steady-state conditions, thereby facilitating tolerance induction via the skin. In addition, skin inflammation has been shown to increase LN-directed Treg homing (Tomura *et al.*, 2010) and triggers reverse circulation of Tregs from LNs to the skin (Matsushima and Takashima, 2010). This mechanism could contribute to the loss of epicutaneous priming against KLH. In this context, our findings that repetitive antigen contact increases Treg percentages and numbers in the skin (Figure 5a and b, denoted by $) but fails to do the same in the lung would support this hypothesis. Single contact with the secondary antigen was not sufficient to increase Treg percentages or numbers, suggesting that the repetition of the treatment played a decisive role for tolerance induction in the skin

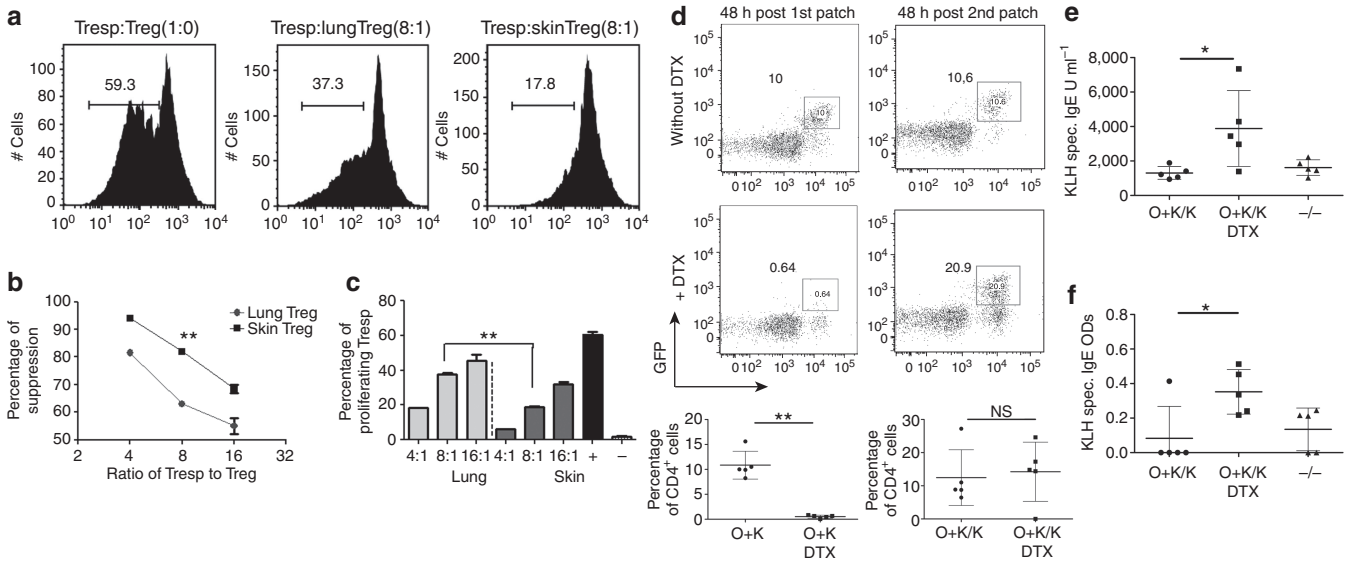


Figure 6. Skin regulatory T cells (Tregs) confer enhanced suppression compared with lung Tregs, and their depletion restores collateral priming in the skin. (**a**) Representative cytometric analyses suppression assay. responder T cells (T_{resp}) alone (left), in co-culture with lung-draining lymph nodes (dLNs) Tregs (middle) or skin dLN Tregs (right) at a ratio of 8:1 T_{resp}:Tregs for 72 hours. (**b** and **c**) Percentage of suppression and proliferation of T_{resp} co-cultured with lung versus skin dLN Tregs at different ratios for 72 hours. + denotes Only T_{resp} Tregs, – denotes T_{resp} not stimulated. Representative experiment from $n=3$. (**d**) Depletion and repopulation control of Tregs in blood samples by flow cytometry ($CD4^+GFP^+Foxp3^+$) of diphtheria toxin (DTX)- and non-treated depletion of regulatory T cells (DEREG) mice. (**e** and **f**) Depletion of Tregs during first patch restores keyhole limpet hemocyanin (KLH)-specific IgG1 and KLH-specific IgE responses in sera. $n=5$ animals per group. –/–: PBS patch. $^*P>0.05$ and $^{**}P>0.01$.

(Figure 5a and b, K only group: -/K). Our experimental groups, however, cannot clarify whether Treg recruitment to the skin is driven by repetitive antigen contact or the effects of patching alone, as we did not include a "patch only" group without antigen.

The composition and function of antigen-presenting cells differ considerably between the lung and the skin (Liu and Nussenzweig, 2010; Igyarto *et al.*, 2011). For example, dermis-derived $CD103^-$ dendritic cells have been shown to induce Tregs (Guilliams *et al.*, 2010). Furthermore, Langerhans cells induce tolerance in a contact hypersensitivity model (Gomez de *et al.*, 2012) and have no counterpart in the lungs. On the other hand, persistent activation of airway dendritic cells in the lungs during the "post inflammatory stage" has been implicated in polysensitization (van Rijt *et al.*, 2011). It seems conceivable that differences in antigen-presenting cell composition and function confer the striking differences we observed in terms of secondary (poly-) sensitization, an issue we will need to address in future studies.

Our results do not concur with the clinical observation that polysensitization also occurs in patients with AD (Ciprandi *et al.*, 2008). Two major differences in pathophysiology might underlie the discrepancy between clinical observations and our data: (i) skin barrier dysfunction has an important role in the development of AD (Oyoshi *et al.*, 2010; De *et al.*, 2012), whereas tolerance induction through administration of an antigen via the EC route crucially depends upon an intact skin barrier (Mondoulet *et al.*, 2012). (ii) Skin lesions of an AD individual are typically colonized with *Staphylococcus aureus* (Breuer *et al.*, 2000, 2002). Disruption of skin barrier function and addition of staphylococcal super-antigens could lead to a skin milieu more conducive to sensitization in our model, which we need to test in further studies.

Epicutaneous immunotherapy with allergen has been demonstrated as being a potential alternative, needle-free, and self-administrable treatment route for subcutaneous immunotherapy in both animal and human studies (reviewed in Senti *et al.* (2014)). Our findings contribute to this therapeutic application by demonstrating that skin Tregs increase with repetitive EC antigen exposure and, furthermore, that there are functional differences between skin and lung Tregs after repetitive local antigen exposure. Similar to Dioszeghy *et al.* (2014), we showed that depletion of Tregs restores sensitization, confirming their crucial role for tolerance induction in the skin. These findings promote the concept of epicutaneous immunotherapy with allergen and suggest that repetitive EC contact leads to local induction of Tregs, which can prevent sensitization toward new allergens through their potent suppressive capacity. Our data could provide an important basis for superior fine tuning of epicutaneous immunotherapy with allergen-containing therapies, with an aim of improving the Treg induction and suppressive capacity, and to potentially prevent polysensitization in mono-sensitized patients.

MATERIALS AND METHODS

Animals

Six- to eight-week-old female BALB/c mice were purchased from Charles River Laboratory (Charles River, Lengefeld, Germany). DEREG (depletion of regulatory T cells) mice (Tg(Foxp3-DTR/EGFP) 23.2Spar; Lahl *et al.*, 2007) on BALB/c mice background were bred in-house. All experimental procedures were performed according to

the local guidelines of the animal welfare law, Landesamt für Verbraucherschutz und Lebensmittelsicherheit (protocol 09-1664).

Titration of sub-immunogenic doses

Naive BALB/c mice were sensitized IP on day 0 and day 7: 20 μg of OVA (grade V; Sigma-Aldrich, Munich, Germany) or KLH (with 1.5 mg alum; Sigma-Aldrich), with negative control groups receiving alum in PBS. In the skin groups, mice were challenged by EC patches from day 18 to 22 and from day 38 to 42 with the help of a circular water proof band-aid (23 mm; Hansaplast, Beiersdorf, Germany) in the indicated doses (Figure 1a and b). The band-aid was placed on the shaved back skin and glued with the help of an adhesive (Mastisol, Eloquest Healthcare, Ferndale, MI). In the lung groups, mice were challenged IN on day 18, 19, and 20 and re-challenged on day 35, 36, 39, and 40 with the indicated doses (Figure1c and d). Killing was performed on day 42 for both groups.

Collateral priming protocol

Priming and challenge were performed as outlined above, with the doses as shown in Figure 2a. The positive groups (O/O) received OVA in both challenges; the collateral priming (O+K/K) groups received OVA and KLH (Sigma-Aldrich) in the first and KLH in the second challenge.

Sub-inflammatory doses (i.e., doses not leading to primary sensitization) and challenge doses had been titrated for each organ system in previous experiments (Figure 1) and are indicated in Figure 2a. All mice were killed and analyzed on day 42 (Figure 1). For cross-over experiments sensitization, challenges, and euthanasia were performed as shown in Figure 2a with the following antigen doses: IP sensitization: 20 μg of KLH (Sigma-Aldrich); challenges: lung group: KLH 25 μg+OVA 5 μg (grade V; Sigma-Aldrich) for the first challenge, followed by OVA 25 μg for second challenge; skin group: KLH 100 μg+OVA 0.25 μg for the first challenge, followed by OVA 100 μg for the second challenge.

In the depletion experiment, DEREG mice were submitted to the EC collateral priming group protocol (Figure 2a, negative controls receiving PBS patches), with 25 $\mu g\,kg^{-1}$ bodyweight IP injection of diphtheria toxin 24 hours before and after first patch application.

Analysis of BAL fluid

BAL inflammatory cells were obtained and analyzed as described previously (Dittrich *et al.*, 2008).

Skin histology

Paraffin-embedded skin samples were sectioned (5 μm), stained with hematoxylin and eosin or toluidine blue, and photographs obtained at ×200 using an Olympus BX51 microscope (Olympus, Hamburg, Germany). For mast cell counts, 35 high-power fields from 7 different sections per group were counted in a blinded manner.

Determination of serum antibody concentration

Antigen-specific antibodies in sera were determined by means of ELISA, as described previously (Dittrich *et al.*, 2008).

Lymph node re-stimulation assays

Single-cell suspensions from dLNs (mediastinal and axillary) of individual mice were re-stimulated for cytokine secretion as described previously (Dittrich *et al.*, 2008).

Cytokine ELISA

Cytokines levels of IFN-γ, IL-4, and IL-13 in culture supernatants were determined by means of ELISA DuoSet kits (R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer's instructions.

Flow-cytometric analysis

Cells were stained on ice for surface markers with fluorescently labeled antibodies (α-CD4 (clone RM4-5), α-CD3 (clone 145-2C11, all BD Biosciences, Heidelberg, Germany)) after blocking with anti-FcR (clone 24G2, lab grown) antibody. Consecutively, cells were fixed and permeabilized with the eBioscience Fix and Perm kit (Frankfurt, Germany) according to the manufacturer's protocols. Labeling with fluorescently coupled antibodies was then performed (α-Foxp3 (clone FJK-16s), α-CTLA-4 (clone UC10-4F10-11, all eBioscience, Frankfurt, Germany)). Cells were analyzed on a LSRII (BD Biosciences) flow cytometer in association with FlowJo (Treestar, Ashland, OR) software.

Suppression assay

Overall, 96-well round bottom plates were coated with antibodies overnight at 37°C (α-CD3 (clone 2C11), α-CD28 (clone 37.51, both lab grown)). Prior to seeding cells, plates were washed with sterile PBS. Splenic CD4+ T cells (T_{resp}) were isolated as described previously (Dittrich *et al.*, 2008) and labeled with VPD450 dye (BD Biosciences) according to the manufacturer's instructions. T_{resp} were co-cultured with CD4+GFP+ cells sorted on a FacsAria from mediastinal or axillary LNs of 10–15 DEREG mice, which had been submitted to the treatment protocol for the EC or IN collateral priming group. Purity of the sorted CD4+GFP+ cells was assessed by means of flow cytometry (Supplementary Figure S2 online). Ratios of T_{resp} to GFP+/Tregs were as indicated, 1 being 4×10^4 cells. Culture conditions were set up in triplicates. Analysis of T-cell proliferation via flow-cytometric dilution of VPD450 dye was performed on day 3. Percentage of suppression was calculated as follows: 100 − [100 × (percentage of proliferation of sample/percentage of proliferation of positive control)].

Statistical analysis

For statistical analysis, *t*-tests or one way analysis of variance in conjunction with Bonferroni's multiple comparison tests was performed with the GraphPad Prism software (Graph Pad Software, La Jolla, CA) to determine statistical differences between means or proportions between two groups of data.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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